Selective elution of target protein from affinity resins by a simple reductant with a thiol group

Miyuki Mabuchi a,b, Masayuki Haramura c, Tadashi Shimizu a, Tomoyuki Nishizaki b, Akito Tanaka a,⇑

a Department of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatojima, Chuo-ku, Kobe 650-8530, Japan
b Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, 1-1 Makogawa-cho, Nishinomiya, Japan
c Discovery Science & Technology Department, Chugai Pharmaceutical Co., Ltd, 200 Kajiwara, Kamakura, Kanagawa 247-8530, Japan

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ABSTRACT

We have made a chance discovery of selective elution of a specific binding protein from affinity resins by mixing them with aqueous solutions of a widely used reductant, 2-mercaptoethanol (2ME), under mild conditions. Our studies suggest this phenomenon would be generic, and could be a powerful method for identification of a specific binding protein. We here exhibit the experimental conditions and successful examples in which target proteins of benzensulfonamide and FK506 were selectively eluted from affinity resins bearing these compounds, while non-specific ones remained.

The identification of target proteins for bioactive compounds such as drugs, natural products and toxins, is an essential component of modern pharmaceutical sciences and chemical biology. It is, therefore, important to identify proteins that specifically bind to such bioactive compounds. While affinity chromatography matrices bearing bioactive compounds are one of the major tools for achieving this goal,1,2 it is often difficult to identify specific binding proteins on affinity resins owing to a preponderance of non-specific binding proteins.3 Selective elution of the desired protein using an aqueous solution of the compound, the so-called ‘ligand elution’ method, is effective for this purpose (Fig. 1A). However, because of low solubility of the compound, this approach is not commonly applicable to hydrophobic compounds, even though these are often attractive for medicinal chemistry and chemical biology. In such cases, efforts to obtain a water-soluble derivative maintaining the bioactivity is required, which often limits its application. A slow dissociation rate of the protein from affinity resins also impairs its application coverage (Fig. S1). Development of a more versatile method has therefore been required.

Currently, there are various alternative methods for identifying specific binding proteins among retained proteins on affinity resins. One is a comparative analysis between binding proteins on affinity resins bearing an ‘active’ compound with those bearing an ‘inactive’ one that has similar structure and less bioactivity.4 However, this approach is also restricted due to the lack of an ‘inactive’ compound. Another method is the competition method that is now widely used, in which an active compound is added to disturb the specific interaction,5 but this method is also limited by the low solubility of a compound. We have developed an alternative method, called the serial affinity chromatography (SAC) method, to distinguish specific binding proteins from non-specific absorptions.5 Due to the SAC method having few limitations, target identifications of bioactive compounds extend to a wide range of synthetic compounds including orally active ones. Furthermore we have developed a novel poly(methacrylate) solid material for affinity resins, AquaFirmus™,6 which, like Toyopearl™, is chemically stable under synthetic conditions and, like AffiGel™, is hydrophilic enough to reduce the non-specific protein absorptions. Continuous study led us to a chance discovery of selective elution of specific binding proteins by mixing with an aqueous solution of a widely used reductant, 2-mercaptoethanol (2ME) (Fig. 1B). We here show the experimental details with successful examples.

BSA (1a) and its derivatives are known as specific binding inhibitors of CAII with a $K_d$ of 0.32–1.25 µM,9 and are often used as model compounds for affinity resins.5 We immobilized a BSA derivative (1b) on AquaFirmus™ to give BSA-affinity resins (3a) (Scheme 1), and mixed them with a lysate obtained from rat brain.10 CAII was successfully identified as a specific binding protein by the
Almost the same, suggesting the importance of the thiol moiety. According to this result, the elution ability of 2ME and DTT were observed. Aqueous solutions of DTT, similarly to 2ME, were also tested in this study. A small amount of CAII was hardly eluted when mixed with aqueous solutions of other small compounds such as methanol (MeOH), ethanol (EtOH), ethylene glycol, n-propanol (n-ProOH), isopropanol (IPA), acetone, acetone, and dimethylsulfoxide (DMSO), indicating structure specificity of 2ME. In order to assess the application range of the above 2ME elution, we evaluated the concentration dependency of the selective elution. A small amount of CAII was also selectively eluted from 3a by mixing with an aqueous solution of 10% 2ME solution in the lysate buffer, while non-specific binding proteins remained on 3a. Interestingly, CAII was hardly eluted when mixed with aqueous solutions of other small compounds such as methanol (MeOH), ethanol (EtOH), ethylene glycol, n-propanol (n-ProOH), isopropanol (IPA), acetone, acetone, and dimethylsulfoxide (DMSO), indicating structure specificity of 2ME.

Next we carried out a time-dependency test (Fig. 3). CAII was obviously eluted from 3a even at 10 min after mixed with the 10% 2ME solution at room temperature (rt), and reached a plateau after 30 min, remaining stable until 120 min later. But elution of non-specific binding proteins and decomposition of CAII were observed after mixed with the 10% 2ME solution at 50 °C for 60 min. These results showed elution by the 10% 2ME solution at rt was suitable for the purpose. Compound 3a was repeatedly mixed with lysate after treatment with 10% 2ME and lysate buffer (Fig. S2) because the elution was suspected of decomposing the affinity resins under the conditions. By taking this step we ensured that the results were not due to decomposition of affinity matrices, but to selective elution of the protein. CAII has a Zn ion at its catalytic center, coordinated by three imidazoles of His residue (Fig. S3). BSA immediately binds to this Zn ion. There was therefore a possibility that CAII was eluted by substitution of imidazoles to the thiol moieties of 2ME or DTT. If so, the application of this method was highly restricted.

**Figure 1.** Selective elution of specific binding protein, CAII, from BSA-affinity resins (3a). After 3a was mixed with lysate obtained from rat brain, each of the binding proteins was eluted under the conditions indicated. Eluted proteins were subjected to SDS-PAGE, and stained with CBB. (A) CAII was selectively eluted by mixing with a 0.1 mM BSA aqueous solution in lysate buffer (0.25 M sucrose, 0.3 mM diethylthiocarbamate, 25 mM Tris-HCl pH 7.5) at rt for 10 min, the so-called ‘ligand elution’ method. Then, remaining proteins were completely eluted by mixing with SDS sample buffer (Nacalai Tesque Inc., cat. 30566-22, containing 4% (w/v)-SDS, 20% (v/v)-glycerol, 0.01% (w/v)-BB, 10% (v/v)-2ME, 0.125 M Tris pH 6.8) at rt for 10 min. (B) Proteins were eluted with 10% of the indicated organic solvent in the lysate buffer under the same conditions. Remaining proteins were also eluted by the SDS sample buffer. (C) The amount of eluted CAII were measured by a KODAK 1D software (version 3.6.1).

**Figure 2.** Concentration dependency of 2ME and DTT for the elution of CAII from 3a. After 3a was mixed with rat brain lysate for 45 min, proteins were eluted with the lysate buffer containing the indicated percentage of 2ME or DTT for 10 min. Proteins were stained by CBB.

**Figure 3.** Time dependency of CAII elution from 3a. After 3a was mixed with rat brain lysate for 45 min, protein was eluted with 10% 2ME solution for the indicated time at rt, or 60 min at 50 °C. Proteins were stained by CBB. Densitometry results was also shown (raw data was shown in Table S1).
Therefore, we next applied this method to identifying the non-metal target protein. FK506 (2a) is an immunosuppressive drug that targets a non-metal protein, FKBP12 (an FK506 binding protein) (Fig. S3), with a $K_d$ of 0.4 nM.14 The structure and functions of the complex of FK506 and its four target proteins (FKBP12, calcineurin A (CnA), calcineurin B (CnB), and calmodulin (Calm)), have been well-characterized at the molecular level.15 Introduction of a linker moiety onto FK506 to connect it to the resins was carried out on a hydroxyl group at the 32 position of FK506 (2b) because crystal structure studies indicate this position is not involved in the specific binding.16 In order to examine 10% 2ME could elute specific target proteins of FK506, FK506-affinity resins (4a) mixed with lysate from rat brain at 4 °C for 45 min were washed with lysate buffer, and mixed with the 10% 2ME solution, and then remaining proteins on resins were completely eluted by mixing with SDS sample buffer (Fig. 4). FKBP12, CnB, and Calm were selectively eluted, and confirmed as specific binding protein by the SAC method.17 Furthermore to ascertain the existence of the last component of the target complex, CnA, a silver-staining was performed on the same gel, which showed that CnA was also eluted, and identified it as a specific binding one (Fig. 4B). These results showed the effectiveness of selective

![Scheme 1. Synthesis of affinity resins bearing BSA or FK506.](image)

**Figure 4.** Target specific elution of FK506 using the 10% 2ME elution method from 4a. After mixing with rat brain lysate for 45 min, binding proteins were eluted by mixing with 10% 2ME in lysate buffer. In order to elute whole binding proteins, the resulting 4a was mixed with SDS sample buffer for 10 min. Identification of specific binding proteins was carried out by the SAC method,5 in which specific binding protein was shown as a protein which markedly decreased between the first mixture 4a (SAC-1) and second one (SAC-2). (A) The known target proteins, FKBP12, calcineurin B (CnB), and calmodulin (Calm), were specifically eluted by mixing with 10% 2ME solution. These proteins were obviously decreased in SAC-2, and therefore confirmed as specific. On the other hand, the known non-specific binding proteins were eluted only by mixing with SDS sample buffer. (B) Calcineurin A (CnA) was identified as a specific binding protein only at the silver-staining level (enlarged view). (C) Each target protein was confirmed by Western blotting analysis.
elution by 2ME solutions for the identification of non-metal target proteins as well as metal ones.

Finally we applied this method to other commercially available solid materials for affinity chromatography, Toyopearl™ and Affigel™ (Fig. 5). Toyopearl™ is relatively hydrophobic and Toyopearl™ bearing BSA (3b) captured a large amount of tubulin and other non-specific binding proteins, some of which were eluted by 10% 2ME solution along with CAII. Therefore 10% 2ME elution was not effective in this case, while elution by 5% 2ME was acceptable for the purpose. The reason for this non selective elution is not clear. Affigel™, consisting of agarose derivatives, is one of the most popular matrices for affinity chromatography, and is hydrophilic enough to reduce non-specific protein absorptions as effectively as AquaFirmus™. Mixing Affigel™ bearing BSA (3c) with 5% or 10% solution afforded selective elution of CAII, and little amount of CAII was left on the resins. These results exhibited that using Affigel™ resins in this method is also effective for the purpose, but using Toyopearl is less so.

BSA and FK506 on affinity resins were thought to specifically bind to CAII and FKBP12, respectively, maintaining active conformation. Because CAII that was eluted by mixing with 10% 2ME lost its binding capability to BSA-affinity resin after removal of 2ME by dialysis (Figs. S4 and S5), they were denatured in the solution,18 and results indicated that 2ME denatured binding CAII with its active conformation and selectively removed from affinity resin.18 We thought the loss of binding ability was not caused by conformation changes created by Cys residues in the protein forming disulfide with 2ME because this redox was often used in lystate buffers and their Cys residues are positioned on the surface.19 On the other hand, non-specific binding proteins, which are thought to stick mostly to solid materials with denatured forms, were little eluted by the 2ME mixture. These results indicated that 2ME could denature active protein on the affinity resin by associating non-specifically and could elute them, but had no ability to slip off the non-specific binding protein (Fig. S6).

In conclusion, we made a chance discovery of selective elution of CAII from BSA-affinity resins by mixing with an aqueous solution of 2ME at rt for 10 min, while little effect was observed by solutions of other similar compounds (Fig. 1). This effect was observed in a widely dynamic-range (Figs. 2, 3 and 5). Utilization of this method enables selective elution of target proteins of FK506 as well (Fig. 4). These results indicated this method could be effective for a wide range of bioactive compounds including hydrophobic bioactive compounds, for which the traditional methods of ‘ligand elution’ or competition would not be appropriate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.053.

References and notes

10. Synthesis of affinity resins and preparation of lystate from rat brain were shown in previous paper.10
11. Typical procedure for elution of proteins by 10% 2ME solution. Lystate from rat brain (1.0 ml) is stirred gently with BSA-affinity resin (3a, 10 μl) at 4 °C for about 45 min, and then precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resulting resins are washed with lystate buffer (200 μl), and resuspended in 30 μl of lystate buffer containing 1 mM BSA, shaken at 25 °C for 10 min. After centrifugation for 1 min, the supernatant is subjected to SDS-PAGE. For elution of remaining proteins, the resulting resins are mixed with 30 μl of SDS sample buffer (Nacalai Tesque Inc., #30566-22, containing 4% (w/v)-SDS, 20% (v/v)-glycerol, 0.01% (w/v)-BB, 10% (v/v)-2ME, 0.125 M Tris pH 6.8) at room temperature for 10 min. The resulting bands were stained with CBB.
13. There was another possibility that the SS bond of cystine in rat’s CAII was cleaved by the 2ME and CAII was eluted because rat CAII also has three Cys residues. However, 3D structure analysis indicates this speculation is unlikely because all Cys residues are located on surface and they do not make SS bonds (see Fig. S3). Figure S3 also indicated that addition of 2ME or DTT to the CAII residues barely perturbed the structure of the proteins.
17. It is known that Cys and Calm are shown at the same position under the SDS– PAGE conditions.
18. Commercially available human CAII (sigma, #CG165) lost its binding ability to 3a in 1% and 10% 2ME in the lystate buffer, while it bound to 3a in 0.1% 2ME solution (Fig. S5). Because stoichiometric amounts of 2ME in 0.1% 2ME solution were much higher than those of CAII in the solution, most of Cys in CAII reacted with 2ME and made disulfide. Therefore the loss of binding-ability of CAII was not caused by Cys residues forming disulfide in the protein mixed with 2ME.